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for

NOVEL IMMUNOTHERAPY

of

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NOVEL IMMUNOTHERAPY

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Background of the Invention

[001] While treatment of disease has progressed far in the last century, many current treatments leave much to be desired when considering the overall welfare of the patient. For instance, currently, chemotherapy and radiation treatment remain the most widely used forms of cancer treatment known. However, such
10 treatments are generic in their attack on the patient's system, attacking both healthy as well as diseased tissues and systems.

[002] Many pathogenic agents, such as cancer cells, for example, use naturally occurring cellular surface receptors, primarily integrins, not only to invade healthy tissue but also for motility within the host body, which, in the case of cancer,
15 can lead to metastasis. Integrins are part of a large family of cell adhesion receptors that are involved in cell/extracellular matrix as well as cell/cell interaction. Integrins are the main method that cells utilize to bind to and respond to the extracellular matrix. Functionally, integrin receptors are composed of two transmembrane glycoprotein subunits, an α subunit and a β subunit. Presently, 16 α and 8 β
20 subunits have been identified.

[003] Pathogens which contain and utilize integrin receptors for interaction with the extracellular matrix or cells of a host can exhibit highly efficient invasion of and motility within an organism due to the nature of the integrin/ligand interactions. Specifically, individual integrin receptors bind their ligands with low affinity (on the
25 order of 10^{-6} to 10^{-9} liters/mole), however, they also exist on cell surfaces in very high concentration, generally 10 to 100 times greater than other types of cell-surface receptors. Following suitable stimulation, integrins on a cell surface will cluster and form hemidesmosomes which can provide a focal contact for adhesion. The combined weak affinities of the multiple integrins at the focal contact can give rise to
30 a spot on the cell surface with suitable adhesive capacity to form an adherence to the ligand. This binding motif provides a method for a single integrin-containing cell to bind simultaneously but weakly to a large number of matrix molecules while still

maintaining the ability to explore the cellular environment. The low affinity integrin/ligand binding motif thus provides an efficient route for integrin-containing pathogens to bind to and invade healthy cells while still maintaining cell motility for further invasion.

5 [004] What is needed in the art are novel treatment methods for disease that can specifically target the pathogens of the disease. Specifically, what is needed in the art are treatment methods that can interfere with the binding processes of the pathogens and prevent initial invasion of healthy cells and motility of the pathogens within the body. Additionally, what is needed in the art is a method to specifically
10 target and bind pathogens with disease fighting agents, such as chemotherapy agents, while not grossly interfering with the healthy systems and tissue that the disease has not yet affected.

Summary of the Invention

 [005] According to one embodiment of the present invention, a therapeutic
15 composition for treatment of disease is disclosed. The therapeutic composition can include a polypeptide capable of binding to at least one of $\alpha 6\beta 1$ integrin receptor and $\alpha 6\beta 4$ integrin receptor, wherein the polypeptide comprises the G domain of the laminin-5 $\alpha 3$ chain or a fragment, mutant, homolog, ortholog, analog, or allele thereof. The therapeutic composition also includes a pharmaceutically compatible
20 carrier for the polypeptide.

 [006] In one particular embodiment, the polypeptides of the present invention can comprise the polypeptide as disclosed in SEQ ID NO:2, or a fragment, mutant, homolog, ortholog, analog, or allele thereof. Optionally, the polypeptides of the present invention can comprise the polypeptide as disclosed in SEQ ID NO:4 or
25 SEQ ID NO: 6 or fragments, mutants, homologs, orthologs, analogs, or alleles of such. In one embodiment, the polypeptide can have at least 70% sequence identity with the reference sequence, i.e., SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

 [007] The therapeutic composition of the present invention can be in any suitable form. For instance, the composition can be a solid or a liquid composition.

In various embodiments, the pharmaceutically compatible carrier can include a gelatin, water, an oil, or a sustained release matrix.

[008] The compositions of the present invention can also include other therapeutic agents, in addition to the disclosed polypeptides. For instance, the
5 compositions can include one or more chemotherapeutic agents or radioactive agents for treatment of a disease, such as a cancer.

[009] In one embodiment, the therapeutic agent of the present invention can be a fused or chimeric polypeptide agent. According to this embodiment, the therapeutic agent can include a first component comprising a polypeptide capable of
10 binding to at least one of $\alpha 6\beta 1$ integrin receptor and $\alpha 6\beta 4$ integrin receptor, wherein the polypeptide comprises the G domain of the laminin-5 $\alpha 3$ chain or a fragment, mutant, homolog, ortholog, analog, or allele thereof. The agent can also include a second component that is chemically bound to the first component. The second component can be any agent for use in the treatment of the disease. For instance,
15 the second component can contribute to the destruction or neutralization of the pathogen bound by the polypeptides of the invention.

[010] The second component can be a protein or a non-protein agent, as desired. For example, the second component can be cytokines, whole antibodies or fractions thereof, cell-surface receptors, ligands for cell-surface receptors, or any
20 suitable organic molecules.

[011] The disclosed invention is also directed to the nucleotide sequences that encode the therapeutic agents. In one particular embodiment, the disclosed invention is directed to an isolated polynucleotide including a first nucleotide sequence encoding the disclosed polypeptides and a second nucleotide sequence
25 that encodes a polypeptide for use in the destruction or neutralization of the pathogens that can be bound by the polypeptides of the invention.

[012] In various embodiments, the first nucleotide sequence can have at least about 70% sequence identity with SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:
5.

[013] The isolated polynucleotide can, in one embodiment be operably linked to an expression control sequence.

[014] The invention is also directed to a host cell transformed with the disclosed polynucleotides. A host cell can be, for example, a bacterial, yeast,
5 mammalian, insect, or plant cell.

[015] The present invention is also directed to methods of treating a disease, such as, for example, breast cancer, with the disclosed therapeutic agents. In general, the methods include contacting a pathogen that includes $\alpha 6\beta 1$ integrin receptors and/or $\alpha 6\beta 4$ integrin receptors on the surface thereof with the therapeutic
10 agents as herein described. Upon contact, the therapeutic agents can then bind to the pathogen.

[016] For example, the method can be carried out *in vivo* and the therapeutic agent can contact the pathogen via a suitable pharmaceutically acceptable administration system. The binding of the agent to the pathogen can mask the
15 integrin receptors of the pathogen and thus prevent the pathogen from binding to the extracellular matrix of the host. In certain embodiments, the method can also include the delivery of a second component of the therapeutic agent to the pathogen, the second component aiding in the destruction or neutralization of the pathogen.

[017] Pharmaceutically acceptable administration systems can include, for example, parenteral systems, oral systems, and sustained release systems.

Brief Description of the Figures

[018] A full and enabling disclosure of the present invention, including the best mode thereof, to one of ordinary skill in the art, is set forth more particularly in
25 the remainder of the specification, including reference to the accompanying figures, in which:

[019] Figure 1 depicts the nucleotide (SEQ ID NO: 1) and the amino acid (SEQ ID NO: 2) sequences of the complete globular domain G-1 to G-5 of the *Rattus norvegicus* laminin-5 $\alpha 3$ chain;

[020] Figure 2 depicts the nucleotide (SEQ ID NO: 3) and the amino acid (SEQ ID NO: 4) sequences of the globular subdomains G-1 through G-3 of the *Rattus norvegicus* laminin-5 α 3 chain;

5 [021] Figure 3 depicts the nucleotide (SEQ ID NO: 5) and the amino acid (SEQ ID NO: 6) sequences of the G-3 subdomain of the *Rattus norvegicus* laminin-5 α 3 chain;

[022] Figure 4 depicts a restriction map for plasmid 5C5 and plasmid 3' α 3 utilized in the Examples;

10 [023] Figure 5 is a table depicting cloning and expression vectors constructed and utilized in Example 1; and

[024] Figures 6-9 graphically illustrate results obtained in Examples 2-5, respectively.

Definitions of Terms

15 [025] "Polypeptide" is herein defined to indicate a molecular chain of amino acids and does not refer to a specific length of the product. Thus, peptides, oligopeptides and proteins are included within the definition of polypeptide. This term is also intended to include polypeptides that have been subjected to post-expression modifications such as, for example, glycosylations, acetylations, phosphorylations and the like.

20 [026] For purposes of this disclosure, the term "protein" is herein defined to include any molecular chain of amino acids that is capable of interacting structurally, enzymatically or otherwise with other proteins, polypeptides or any other organic or inorganic molecule.

25 [027] The term "fragment" in reference to a protein or polypeptide is herein defined as an amino acid sequence of that protein that is shorter than the entire protein, but comprising at least about 25 consecutive amino acids of the full polypeptide.

30 [028] For purposes of this disclosure, an "ortholog" is herein defined to be a nucleotide or polypeptide sequence with similar function to a nucleotide or polypeptide sequence in an evolutionarily related species. Loci in two species are

said to be "orthologous" when they have arisen from the same locus of their common ancestor. Orthologous polynucleotide sequences exist at loci in different species that are sufficiently similar to each other in their nucleotide sequences to suggest that they originated from a common ancestral sequence. Orthologous sequences arise when a lineage splits into two species, rather than when a sequence is duplicated within a genome. Proteins that are orthologs of each other are encoded by genes of two different species, and the genes are said to be orthologous.

[029] The term "mutant" is herein defined to be a polypeptide that includes any change in the amino acid sequence relative to the amino acid sequence of the reference polypeptide. Such changes can arise either spontaneously or by manipulations including those chemical derivatives brought about by chemical energy (e.g., X-ray), other forms of chemical mutagenesis, by genetic engineering, or as a result of mating or other forms of exchange of genetic information. Mutations include, e.g., base changes, deletions, insertions, inversions, translocations, or duplications. Mutants may or may not also comprise additional amino acids derived from the process of cloning, e.g., amino acid residues or amino acid sequences corresponding to full or partial linker sequences. Mutants/fragments of the polypeptides of the present invention can also be generated by PCR cloning, or by *Pseudomonas* elastase digestion, as described by Mariyama, M. et al. (1992, J. Biol. Chem. 267:1253-1258).

[030] The term "homolog" is herein defined to describe two nucleotide or polypeptide sequences that differ from each other by substitutions that do not effect the overall functioning of the polypeptide. For example, when considering polypeptide sequences, homologs include polypeptides having substitution of one amino acid at a given position in the sequence for another amino acid of the same class (e.g., amino acids that share characteristics of hydrophobicity, charge, pK or other conformational or chemical properties, e.g., valine for leucine, arginine for lysine). Homologs also include polypeptides and nucleotide sequences including one or more substitutions, deletions, or insertions, located at positions of the

sequence that do not alter the conformation or folding of the polypeptide to the extent that the biological activity of the polypeptide is destroyed. Examples of possible homologs include polypeptide sequences including substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for one another; the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, or between threonine and serine; the substitution of one basic residue such as lysine, arginine or histidine for another; the substitution of one acidic residue, such as aspartic acid or glutamic acid for the another; or the use of a chemically derivatized residue in place of a non-derivatized residue, as long as the homolog polypeptide displays substantially similar biological activity to the reference polypeptide, and in particular the ability to be recognized and be bound by $\alpha 6\beta 1$ and/or $\alpha 6\beta 4$ integrin receptors.

[031] The term "analog" is herein defined to be a non-natural molecule substantially similar to either the entire reference protein or polypeptide, or a fragment or allelic variant thereof, and having substantially the same or superior biological activity. The term "analog" is intended to include derivatives (e.g., chemical derivatives, as defined above) of the biologically active polypeptide, as well as its fragments, mutants, homologs, orthologs, and allelic variants, which derivatives exhibit a qualitatively similar agonist or antagonist effect to that of the unmodified polypeptide.

[032] The term "allele" of a polypeptide is herein defined to be a polypeptide sequence containing a naturally-occurring sequence variation relative to the polypeptide sequence of the reference polypeptide. Similarly, an allele of a polynucleotide encoding the polypeptide is herein defined to be a polynucleotide containing a sequence variation relative to the reference polynucleotide sequence encoding the reference polypeptide, where the allele of the polynucleotide encoding the polypeptide encodes an allelic form of the polypeptide.

[033] "Operably linked" refers to a situation wherein the components described are in a relationship permitting them to function in their intended manner. For instance, a control sequence "operably linked" to a coding sequence is ligated in

such a manner that expression of the coding sequence is achieved under conditions compatible with the control sequence. A "coding sequence" is a polynucleotide sequence which is transcribed into mRNA and translated into a polypeptide when placed under the control of (e.g., operably linked to) appropriate regulatory

5 sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. Such boundaries can be naturally-occurring, or can be introduced into or added to the polynucleotide sequence by methods known in the art. A coding sequence can include, but is not limited to, genomic DNA, mRNA, cDNA, and recombinant
10 polynucleotide sequences.

[034] The term "sequence identity," as used herein, refers to the subunit sequence similarity between two polymeric molecules. For example, the sequence similarity between two polynucleotides or two polypeptides. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, then they
15 are identical at that position. The identity between two sequences is a direct function of the number of matching or identical positions. For example, if half of the positions in two peptide or compound sequences are identical, then the two sequences are 50% identical. The identity between two sequences is a direct function of the number of matching or identical positions. Thus, if a portion of the
20 reference sequence is deleted in a particular peptide, that deleted section is not counted for purposes of calculating sequence identity. For example, when comparing a first polymer including monomers $R_1R_2R_3R_4R_5R_6$ with another polymer including monomers $R_1R_2R_3R_4R_6$, the two polymers have 5 out of 6 positions in common, and therefore would be described as sharing 83.3% sequence identity.

25 [035] The term "pathogen" is herein defined to include any disease causing agent. Pathogens can include disease causing agents that can infect a host from an external source, such as bacteria, fungus, virus, and the like, as well as pathogenic agents arising within the carrier of the disease, including abnormal cells such as cancer cells.

Detailed Description of the Invention

[036] Reference will now be made in detail to various embodiments of the invention, one or more examples of which are set forth below. Each embodiment is provided by way of explanation of the invention, not limitation of the invention. In fact, it will be apparent to those skilled in the art that various modifications and variations may be made in the present invention without departing from the scope or spirit of the invention. For instance, features illustrated or described as part of one embodiment, may be used in another embodiment to yield a still further embodiment. Thus, it is intended that the present invention cover such modifications and variations as come within the scope of the appended claims and their equivalents.

[037] The present invention is generally directed to a novel immunotherapy in which a protein or polypeptide agent is produced that is capable of binding to the surface receptors of a pathogen. Through this binding, the agent can mask the receptors of the pathogen and prevent the binding of the pathogen to healthy host cells. In addition, through this binding, the agent can, in one particular embodiment, be utilized to carry and bind other materials to the pathogen that can encourage the destruction or neutralization of the pathogen. More specifically, the therapeutic agents of the present invention comprise at least a portion of a laminin-5 chain that can be utilized as an immunotherapeutic agent against pathogens that include $\alpha 6\beta 1$ and/or $\alpha 6\beta 4$ integrin receptors on the surface of the pathogen.

[038] Laminin is one of a family of heterotrimer protein complexes formed from various combinations of different α , β , and γ subunit chains. Laminins in general can be found in the basement membranes of the extracellular matrix and interact with other matrix macromolecules to contribute to cell differentiation, movement and maintenance. Laminin-5, comprising an $\alpha 3$ chain, a $\beta 3$ chain, and a $\gamma 2$ chain, is a member of the laminin family that has been shown to function as an adhesion and migration component for certain cells. The terminal portion of the laminin-5 $\alpha 3$ chain, the G domain, is further subdivided into 5 sub-domains, G1, G2, G3, G4, and G5. The G subdomains of the laminin-5 $\alpha 3$ chain have been shown to be necessary for adherence of laminin-5 to cells which have certain receptor

integrins on their cell surface, specifically, cells containing $\alpha 6\beta 1$ and/or $\alpha 6\beta 4$ integrin receptors on the cell surface.

[039] The present invention is generally directed to recognition and utilization of the binding characteristics of the G-domain of the laminin-5 $\alpha 3$ chain. More specifically, according to the present invention, the entire laminin-5 $\alpha 3$ G-domain as well as significant portions of the laminin-5 $\alpha 3$ G-domain have been sequenced and expressed for utilization in novel immunotherapies targeting pathogens containing specific integrin receptors on the surface, specifically, those containing $\alpha 6\beta 1$ and/or $\alpha 6\beta 4$ integrin receptors on the surface.

[040] In one embodiment, therapeutic compositions comprising polypeptides of the disclosed portions of the G domain of the laminin-5 $\alpha 3$ chain have been developed that can be utilized to target and bind to the $\alpha 6\beta 1$ and/or $\alpha 6\beta 4$ integrin receptors on the surface of a pathogen. The present invention is also directed to mutants, homologs, orthologs, analogs, and/or allelic variants of the laminin-5 $\alpha 3$ G domains disclosed herein possessing the ability to be recognized and bound by $\alpha 6\beta 1$ and/or $\alpha 6\beta 4$ integrin receptors.

[041] The laminin-5 of the present invention can be obtained from a variety of sources. For example, while SEQ ID NO: 1-6 are particular to rat laminin-5 G domains (and in particular, *Rattus norvegicus*), other sources of laminin-5 are encompassed by the disclosed invention. Such sources include, but are not limited to mouse laminin-5, *Mus musculus* laminin-5, artificial laminin-5 and human laminin-5.

[042] In one embodiment, therapeutic agents can be developed comprising that portion of the G-domain of the laminin-5 $\alpha 3$ chain that binds to $\alpha 6\beta 1$ and/or $\alpha 6\beta 4$ integrin receptors, which is understood to exist in the G-3 subdomain of the chain. For instance, in one embodiment, the therapeutic agents of the present invention can include polypeptides including all of the G1 – G5 subdomains of the laminin-5 $\alpha 3$ chain as identified in SEQ ID NO: 2 as well as mutants, homologs, orthologs, analogs, and/or allelic variants of the laminin-5 $\alpha 3$ G1-G5 domain as identified in SEQ ID NO: 2.

[043] In other embodiments, the therapeutic agents of the present invention can include only those sub-domains of the laminin-5 $\alpha 3$ chain believed to contain the specific amino acid sequences that bind to the $\alpha 6\beta 1$ and/or $\alpha 6\beta 4$ integrin receptors of the cellular pathogens. For instance, in one embodiment, the therapeutic agents of the present invention can include the G1–G3 subdomains of the laminin-5 $\alpha 3$ chain as identified in SEQ ID NO: 4 as well as mutants, homologs, orthologs, analogs, and/or allelic variants of the laminin-5 $\alpha 3$ G1-G3 subdomains as identified in SEQ ID NO: 4.

[044] In another embodiment, the therapeutic agents of the present invention can include only the G3 subdomain of the laminin-5 $\alpha 3$ chain as identified in SEQ ID NO: 6 as well as mutants, homologs, orthologs, analogs, and/or allelic variants of the laminin-5 $\alpha 3$ G3 subdomain as identified in SEQ ID NO: 6.

[045] Encompassed by the present invention are proteins and polypeptides that have substantially the same amino acid sequence as the laminin-5 $\alpha 3$ G domains as herein disclosed as well as the polynucleotides that encode such. By the term “substantially the same” is meant both the polypeptide and the polynucleotide that encodes such that can be recognized and bound by $\alpha 6\beta 1$ and/or $\alpha 6\beta 4$ integrin receptors. For example, in one embodiment, the nucleotide or amino acid sequence of the present invention can exhibit at least about 70% sequence identity with the reference sequence, at least about 80% sequence identity with the reference sequence, at least about 90% sequence identity, at least about 95% sequence identity, or at least about 97% sequence identity with the reference sequence. Optionally, the polypeptide can be only that small portion of the entire G domain that is recognized and bound by $\alpha 6\beta 1$ and/or $\alpha 6\beta 4$ integrin receptors.

[046] In addition, the presently disclosed invention is directed not only to the disclosed polypeptides and the polynucleotides encoding such, but is also directed to the vectors and host cells containing such polynucleotides. Vectors encompassed by the disclosed invention include any molecules into which pieces of nucleic acid may be inserted or cloned that can transfer the nucleic acids carried thereby into a host cell. In some embodiments of the present invention, vectors may

also bring about the replication and/or expression of the transferred nucleic acid pieces. An exemplary list of suitable vectors can include nucleic acid molecules derived from a plasmid, bacteriophage, or mammalian, plant or insect virus, or non-viral vectors such as ligand-nucleic acid conjugates, liposomes, or lipid-nucleic acid complexes.

[047] In some embodiments of the present invention, the transferred nucleic acid molecule can be operatively linked to an expression control sequence to form an expression vector capable of expressing the transferred nucleic acid. Such transfer of nucleic acids is generally termed transformation, and refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion. For example, direct uptake, transduction, electroporation, or f-mating, as are generally known in the art, can be utilized. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome.

[048] The vector into which the disclosed polynucleotides can be cloned may be chosen because it functions in either a prokaryotic organism, a eukaryotic organism, or both, as desired. An exemplary list of possible vectors include, for example, the pGEM™-T Easy vector, the pYES2 vector, the pPICZ.alpha series of vectors, the pET22b and pET28(a) vectors, and modified pPICZ.alpha series vectors.

[049] Following the cloning of a polynucleotide into a suitable vector, the vector can be transformed into an appropriate host cell. By "host cell" is meant a cell which has been or can be used as the recipient of transferred nucleic acid by means of a vector. Host cells can be prokaryotic or eukaryotic, mammalian, plant, or insect, and can exist as single cells, or as a collection, e.g., as a culture, or in a tissue culture, or in a tissue or an organism. Host cells can also be derived from normal or diseased tissue from a multicellular organism, e.g., a mammal. Host cell, as used herein, is intended to include not only the original cell which was transformed with a nucleic acid, but also descendants of such a cell, which still contain the nucleic acid.

[050] In one embodiment, isolated polynucleotides encoding the disclosed polypeptides can additionally comprise a polynucleotide linker encoding a peptide. Such linkers are generally known to those of skill in the art and can comprise, for example, at least one additional codon encoding at least one additional amino acid.

- 5 Typically the linker comprises one to about twenty or thirty amino acids. The polynucleotide linker can be translated along with the disclosed polynucleotides resulting in the expression of the disclosed polypeptides with at least one additional amino acid residue at the amino or carboxyl terminus of the polypeptide. Importantly, the additional amino acid, or amino acids, do not compromise the
- 10 recognition and binding capability of the polypeptides by $\alpha 6\beta 1$ and/or $\alpha 6\beta 4$ integrin receptors.

- [051] In one embodiment, following insertion of the disclosed polynucleotide into a vector, the vector can be transformed into an appropriate prokaryotic strain and the strain can be maintained under suitable culture conditions for the production
- 15 of the encoded polypeptide.

- [052] In another embodiment of the present invention, a eukaryotic vector can be utilized that comprises a modified yeast vector. According to one particular embodiment, a plasmid can be utilized that contains a multiple cloning site. In addition, the multiple cloning site can have inserted thereto a His.Tag motif, as is
- 20 generally known in the art. Optionally, the vector can be modified to add a restriction site, for example an NdeI site. Such sites are well known to those of skill in the art. Proteins and polypeptides produced according to this particular embodiment can comprise a histidine tag motif (His.tag) comprising one or more histidines, in one embodiment about 5-20 histidines. Of course, any tag should not interfere with the
- 25 desired properties of the proteins and product, namely, the ability for recognition and binding thereto by $\alpha 6\beta 1$ and/or $\alpha 6\beta 4$ integrin receptors.

- [053] In one embodiment of the present invention, the disclosed polypeptides can be synthetically constructed amino acid sequences produced according to conventional methods of chemical synthesis as are generally known to
- 30 those in the art.

[054] Pathogens that can be targeted by the disclosed therapeutic agents can include any pathogens that include $\alpha6\beta1$ and/or $\alpha6\beta4$ integrin receptors on the surface. For example, in some embodiments, the disclosed therapeutic agents can be targeted toward certain cellular pathogens, including cancer cells, and
5 specifically, breast cancer cells, prostate cancer cells, thyroid cancer cells, bladder cancer cells, colorectal cancer cells, intestinal cancer cells, squamous cell carcinomas, neuroblastomas, and fibrotic liver tissue cells.

[055] In addition to the therapeutic agents herein disclosed, the present invention is also directed to methods for treatment of disease utilizing the disclosed
10 therapeutic agents. For example, in one embodiment, the disclosed methods can be utilized for the destruction of primary and secondary cancer or tumor cells by contacting and binding the agents to the pathogenic cells and masking the integrin receptors of the cells, preventing communication between the pathogenic cells and the host and leading to the eventual death of the cancer cells.

[056] In one particular embodiment, the present invention is directed to a
15 method for the inhibition or elimination of metastatic cancer or tumor cells arising from primary tumor sites. According to this embodiment, the therapeutic agents of the present invention can be directed toward a tumor or cancer by use of any suitable pharmaceutically acceptable system and can contact the cancer cells in that
20 targeted area. As the targeted cancer cells include $\alpha6\beta1$ and/or $\alpha6\beta4$ integrin receptors on the surface, the agents of the present invention can bind to the pathogens at the $\alpha6\beta1$ and/or $\alpha6\beta4$ integrin receptors. The binding of the therapeutic agents to the cellular pathogens can mask the receptors of the cancer cells, as discussed above, and motility of the metastatic cell can be prevented. In
25 addition, in certain embodiments of the invention, this masking of the pathogen cannot only prevent the spread of the cancer, but can also destroy the cancerous cells, for example in those cases where the binding of the therapeutic agents to the cancer cells also prevents the cancer cells from obtaining necessary nutrition.

[057] In other embodiments of the invention, treatment with the therapeutic
30 agents can be combined with other known treatment agents or methods to destroy

or treat a disease. More specifically, the polypeptides of the disclosed invention may be used in combination with themselves or other compositions and procedures for the treatment of diseases. For instance, in one embodiment, the disclosed polypeptides can be combined with another treatment agent such as a

5 chemotherapeutic agent. For example, a therapeutic composition of the present invention can include the disclosed polypeptides and a second therapeutic agent such as Vasostatin or anti-alpha 6 integrin monoclonal antibodies with a pharmaceutically compatible carrier. In another embodiment, a disease may be treated conventionally with surgery, radiation, or chemotherapy, and the disclosed
10 polypeptides may additionally be administered to the patient to further treat the disease such as by extension of the dormancy of micrometastases or to stabilize and inhibit the growth of any residual primary tumor.

[058] In another embodiment, the disclosed polypeptides can be combined with other pharmaceutically acceptable excipients in forming therapeutic
15 compositions. The compositions of the present invention may additionally contain other polypeptides or chemical compounds for disease treatment as are generally known in the art. Such additional factors and/or agents may be included in the composition to produce a synergistic effect with the polypeptides of the invention.

[059] In one embodiment of the present invention, the disclosed
20 polypeptides can be chemically combined with secondary materials so as to form a single agent comprising both materials. Generally, the second component of the combination agent can be useful in fighting the disease, for example can aid in destruction or neutralization of the pathogen. For example, in one embodiment, the recognition and binding of the polypeptides to the pathogen can be utilized as a
25 method for delivering the second component directly to the pathogen. For example, the therapeutic agents of the present invention can include fusion polypeptides or chimeric polypeptides comprising the disclosed polypeptides of the laminin-5 $\alpha 3$ G domain, or their fragments, mutants, homologs, orthologs, analogs, and allelic variants, chemically combined with a secondary polypeptide material so as to form a
30 single therapeutic agent. Exemplary secondary polypeptide materials can include,

for example, IL-2, IL-3 IL-15, IL-12, IFN- γ , GM-CSF, CD40, CD40 ligand (CD40L), C3 Complement components, CD80, CD86, FAS, or FAS ligand (FASL). The nucleotide and amino acid sequences of each of these exemplary components are known in the art and can be found in the NCBI GenBank database.

5 [060] In one embodiment, a fusion or chimeric product or polypeptide of the present invention can be produced as a result of recombinant expression and the cloning process as described above, and the polypeptide may be produced comprising additional amino acids or amino acid sequences corresponding to full or partial linker sequences. Alternatively, a fusion or chimeric product of the present
10 invention can be a multimer of a single polypeptide. That is, a polypeptide including one or more repeating sequences of the disclosed polypeptides. In yet another embodiment, the therapeutic agents of the present invention can be fusion and chimeric polypeptides that can be formed of one or more of the different polypeptides as herein disclosed. For example, in one embodiment, a therapeutic
15 agent according to the present invention can include a polypeptide comprising a polypeptide as disclosed according to SEQ ID NO: 2 in combination with one or more polypeptides as disclosed according to SEQ ID NO: 4 and/or SEQ ID NO: 6.

 [061] In yet another embodiment of the present invention, the therapeutic agent can be a fusion or chimeric product in which polypeptides as herein disclosed
20 are chemically combined with other, non-protein secondary components so as to form a single therapeutic agent. For example, the disclosed polypeptides can be combined with additional components such as, for example, superantigens, muramyl dipeptide (MDP), lipopolysaccharide (LPS), or mannose. According to this embodiment, fusion or chimeric therapeutic agents encompassed by the present
25 invention can generally include one or more of the disclosed laminin-5 x 3 α G domain polypeptides linked together with other materials via post-translational modification through covalent bonds such as amide, ester, disulfide or azo bonds, for example.

 [062] In general, methods for treatment of disease utilizing the disclosed
30 agents include contacting the cellular pathogen with a composition comprising the

polypeptides of the invention. For example, in one embodiment, the methods of the disclosed invention can be utilized *in vivo* for treatment of a disease such as cancer. According to this embodiment, a composition including the disclosed therapeutic agents and a pharmaceutically compatible carrier can be delivered to a patient via any pharmaceutically acceptable delivery system. For instance, a composition of the present invention including a pharmaceutically compatible carrier and the disclosed polypeptides may be a solid, liquid or aerosol and may be administered by any known pharmaceutically acceptable route of administration. A non-limiting exemplary listing of possible solid compositions can include pills, creams, and implantable dosage units. An implantable dosage unit can, in one embodiment, be administered locally, for example at a tumor site, or can be implanted for systemic release of the composition, for example subcutaneously. A non-limiting exemplary listing of possible liquid compositions can include formulations adapted for injection subcutaneously, intravenously, intraarterially, and formulations for topical and intraocular administration. Possible examples of aerosol formulations include inhaler formulations for direct administration to the lungs.

[063] The proteins and protein fragments of the disclosed invention can be provided as isolated and substantially purified proteins and protein fragments in pharmaceutically acceptable formulations using formulation methods known to those of ordinary skill in the art. These formulations can generally be administered by standard routes. For example, the combinations may be administered by topical, transdermal, intraperitoneal, intracranial, intracerebroventricular, intracerebral, intravaginal, intrauterine, oral, rectal or parenteral (e.g., intravenous, intraspinal, subcutaneous or intramuscular) route. Osmotic minipumps may also be used to provide controlled delivery of high concentrations of the disclosed polypeptides through cannulae to the site of interest, such as directly into a metastatic growth.

[064] Pharmaceutical compositions for parenteral injection according to the present invention include pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to

use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (e.g., glycerol, propylene glycol, polyethylene glycol and the like), carboxymethylcellulose and suitable mixtures thereof, vegetable oils (e.g., olive oil) and injectable organic esters such as ethyl

5 oleate. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient. Proper fluidity may be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use
10 of surfactants. These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents such as paraben, chlorobutanol, phenol, sorbic acid and the like. It may also be desirable to include isotonic agents such as sugars,
15 sodium chloride and the like.

[065] Prolonged absorption of an injectable pharmaceutical form may be brought about by the inclusion of agents, such as aluminum monostearate and gelatin, which can delay absorption. For example, injectable depot forms can be made by forming microencapsule matrices of the therapeutic agent in biodegradable
20 polymers such as polylactide-polyglycolide, poly(orthoesters) and poly(anhydrides). Depending upon the ratio of therapeutic agent to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Depot injectable formulations can also be prepared by entrapping the therapeutic agents in liposomes or microemulsions which are compatible with body tissues. The
25 injectable formulations may be sterilized, for example, by filtration through a bacterial-retaining filter or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable media just prior to use.

[066] In one embodiment, the therapeutic compositions of the present
30 invention can include pharmaceutically acceptable salts of the components therein,

e.g., those that may be derived from inorganic or organic acids. Pharmaceutically acceptable salts are well known in the art. For example, S. M. Berge, et al.

describes pharmaceutically acceptable salts in detail in J. Pharmaceutical Sciences (1977) 66:1 et seq., which is incorporated herein by reference. Pharmaceutically

5 acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or
10 ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like. The salts may be prepared in situ during the final isolation and purification of the compounds of the invention or separately by reacting a free base function with a suitable organic acid.

Representative acid addition salts include, but are not limited to acetate, adipate,

15 alginate, citrate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, camphorate, camphorsulfonate, digluconate, glycerophosphate, hemisulfate, heptanoate, hexanoate, fumarate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxymethanesulfonate (isethionate), lactate, maleate, methanesulfonate, nicotinate, 2-naphthalenesulfonate, oxalate, pamoate, pectinate, persulfate, 3-
20 phenylpropionate, picrate, pivalate, propionate, succinate, tartate, thiocyanate, phosphate, glutamate, bicarbonate, p-toluenesulfonate and undecanoate. Also, the basic nitrogen-containing groups can be quaternized with such agents as lower alkyl halides such as methyl, ethyl, propyl, and butyl chlorides, bromides and iodides; dialkyl sulfates like dimethyl, diethyl, dibutyl, and diamyl sulfates; long chain halides
25 such as decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides; arylalkyl halides like benzyl and phenethyl bromides and others. Water or oil-soluble or dispersible products are thereby obtained. Examples of acids which may be employed to form pharmaceutically acceptable acid addition salts include such inorganic acids as hydrochloric acid, hydrobromic acid, sulphuric acid and

phosphoric acid and such organic acids as oxalic acid, maleic acid, succinic acid and citric acid.

[067] In one embodiment the treatment method can include use of timed release or sustained release delivery systems as are generally known in the art.

- 5 Such systems can be desirable, for instance, in situations where surgery is difficult or impossible, e.g., situations involving patients debilitated by age or the disease course itself, or where the risk-benefit analysis dictates control over cure. According to this particular embodiment, a sustained-release matrix can include a matrix made of materials, usually polymers, which are degradable by enzymatic or acid/base
- 10 hydrolysis or by dissolution. Once inserted into the body, such a matrix can be acted upon by enzymes and body fluids. The sustained-release matrix desirably is chosen from biocompatible materials such as liposomes, polylactides (polylactic acid), polyglycolide (polymer of glycolic acid), polylactide co-glycolide (co-polymers of lactic acid and glycolic acid) polyanhydrides, poly(ortho)esters, polyproteins,
- 15 hyaluronic acid, collagen, chondroitin sulfate, carboxylic acids, fatty acids, phospholipids, polysaccharides, nucleic acids, polyamino acids, amino acids such as phenylalanine, tyrosine, isoleucine, polynucleotides, polyvinyl propylene, polyvinylpyrrolidone and silicone. Possible biodegradable polymers and their use are described, for example, in detail in Brem et al. (1991, J. Neurosurg. 74:441-6),
- 20 which is hereby incorporated by reference in its entirety.

- [068] When an effective amount of therapeutic agents of the present invention is administered orally, the therapeutic compositions can be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier
- 25 such as a gelatin or an adjuvant. The tablet, capsule, or powder can, for example, contain from about 5 to 95% therapeutic agents of the present invention. In one embodiment, the composition can contain from about 25 to 90% therapeutic agents of the present invention.

- [069] When administered orally in liquid form, a liquid carrier such as water,
- 30 petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil,

or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical

5 composition contains from about 0.5 to 90% by weight of therapeutic agents of the present invention, in one embodiment from about 1 to 50% therapeutic agents of the present invention.

[070] When an effective amount of the agents of the present invention are administered by intravenous, cutaneous or subcutaneous injection, the polypeptides
10 of the present invention can generally be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable polypeptide solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection can contain, in addition to the polypeptides of
15 the present invention, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

20 [071] The dosage of the disclosed polypeptides of the present invention can depend on the disease state or condition being treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. Depending upon the half-life of the disclosed polypeptides in the particular animal or human, the disclosed polypeptides can be administered between
25 several times per day to once a week. It is to be understood that the present invention has application for both human and veterinary use. The methods of the present invention contemplate single as well as multiple administrations, given either simultaneously or over an extended period of time. In addition, the disclosed polypeptides can be administered in conjunction with other forms of therapy, e.g.,
30 chemotherapy, radiotherapy, or other immunotherapy.

[072] The present invention may be better understood with respect to the following examples.

Example 1

General Methodologies for Construction, Expression, and Isolation of Laminin-5- α 3

5 Peptides

[073] Manipulations of DNA were completed according to standard techniques as described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory Press, New York, 1989. Restriction enzymes were purchased from either Promega or New England Biolabs. *Escherichia coli* (*E. coli*) strains DH5 α MCR (Jessee and Bloom, 1988), GM2163 (New England Biolabs), and JM109 (Promega) were used throughout and grown in either L. broth (Lennox, 1955) or Terrific Broth (Tartof and Hobbs, 1987). All cultures were grown at 37°C and liquid cultures were agitated at 250 rpm. Plasmid ligation reactions were performed according to protocols found in Sambrook *et al.* (1989). Plasmid ligations were transformed into *E. coli* competent cells (from strains DH5 α MCR or GM2163) by a heat-shock procedure (Henson, 1984) while transformations into *E. coli* competent cells from strain JM109 were conducted according to the manufacturer's protocol. Plasmid DNA from putative transformants was isolated using a plasmid miniprep system.

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Plasmid construction and transformation

[074] Two partial cDNA clones from rat laminin-5- α 3 cloned into pBluescript SK (Stratagene) were obtained from Northwestern University. Plasmid 5C5 encoded the region of 932-3242 bp of laminin-5 α 3 chain region, and, plasmid 3' α 3 encoded the 3' half of laminin-5- α 3 chain region from 3092-5250 bp. Figure 4 illustrates the restriction map.

[075] Plasmid pHB1 was constructed from PCR amplification of the 5C5 cDNA (see Figure 5). For cloning in the vector pGEM-T Easy, the following synthetic oligonucleotide primers were used for PCR amplification: forward (5'-AATTAACCCTCACTAAAGGG-3') (SEQ ID NO: 7) and reverse (5'-

30

TAATACGACTCACTATAGGG-3') (SEQ ID NO: 8). The addition of Taq polymerase resulted in poly A-overhangs that were added to the PCR products and purified from 1% agarose gel using the Rapid Plasmid Miniprep System (Marligen Biosciences, Inc). The agarose purified product was ligated into pGEM-T Easy. The ligation reaction was carried out for 4 hours at room temperature. *E. coli* strain GM2163 (New England Biolabs) was transformed (Sambrook, et al 1989) with the ligation product. Proper construction of the final plasmid pHB1 was verified by restriction analysis and DNA sequencing on a Licor 4200L sequencer using T7 and SP6 primers.

[076] Plasmid pHB2 was constructed from PCR amplification of the 3' α 3 cDNA. For cloning in the vector pGEM-T Easy, the following synthetic oligonucleotides primers were used for PCR amplification: forward (5'-CCAGACTACTGTGGACAGAGG-3') (SEQ ID NO: 9) and reverse (5'-AAGGGTTCTTCGTGTGTAGGG-3') (SEQ ID NO: 10). Procedures for plasmid construction, transformation and verification were continued as described previously for pHB1.

[077] Plasmid pHB3 was constructed from pHB2 by digesting with *Xba*I, precipitation, followed by a partial digest with *Eco*RI. Aliquots were removed in 10 μ l volumes every 3 minutes followed by the addition of 1 μ l 0.5 M EDTA pH 8.0.

Restriction digest products were viewed on 1.5% agarose gel, and the desired 1761 bp fragment was agarose purified as described above. Cloning vector pYES2 was digested by *Xba*I and *Eco*RI and ligated with the 1761 bp *Xba*I/*Eco*RI partial fragment from pHB2. The ligation reaction was carried out for 24 hours at room temperature and transformed into *E. coli* strain DH5 α MCR. Proper construction was verified by restriction enzyme analysis.

[078] Plasmid pHB4 (containing G1-G5 domains of laminin-5- α 3) was constructed from pHB1 digest with *Sac*I/*Eco*RI. The resulting 822 bp fragment from 5C5 was agarose purified. Plasmid pHB3 was digested with *Sac*I, precipitated, followed by an *Eco*RI partial digest performed as illustrated previously. Restriction digest products were viewed on a 0.7% agarose gel and the desired fragment

containing pYES2 + 1761 bp was agarose purified. The 822 bp fragment from pHB1 was ligated with pYES + 1761 bp (linearized pHB3) for 24 hours at room temperature and transformed in *E. coli* strain DH5 α MCR. Proper construction was verified by restriction enzyme analysis. Internal junctions were sequenced on ABI
5 Prism 3700 DNA Analyzer using the primers: forward (5'-CTACTCAACCAAATGCTCCC-3') (SEQ ID NO: 11) and reverse (5'-GTACTATTCAACCTGACAACCC-3') (SEQ ID NO: 12). Prior to sequencing, dye-terminator removal was completed using Qiagen DyeEx™ 2.0 Spin Kit.

10 Expression vector construction and transformation in *E. coli*

[079] The expression of recombinant laminin proteins was produced using the EasySelect™ *Pichia* Expression Kit from Invitrogen. Cloning of laminin-5- α 3 G domains into the *P. pastoris* expression vector pPICZ α B for the construction of pHB6 was performed in a multi-step procedure. pPICZ α B was digested with *Kpn*I,
15 followed by a T4 DNA polymerase reaction to blunt 3' overhangs, precipitated, digested with *Xba*I and the resulting fragment was purified from a 1% agarose gel. The DNA fragment encoding the regions of G1-G5 of laminin-5- α 3 chain from pHB4 was digested with *Sac*I, followed by treatment with T4 DNA polymerase, digested with *Xba*I and also purified from a 1% agarose gel. The ligation reaction was 24
20 hours at room temperature, followed by transformation in *E. coli* strain JM109 and plated onto low salt LB Zeocin™ plates. Proper construction of the vector pHB6 was verified by restriction analysis and DNA sequencing. The following synthetic oligonucleotides were used: forward AOX1 (5'-GACTGGTTCCAATTGACAAGC-3') (SEQ ID NO: 13) and reverse AOX1 (5'-GCAAATGGCATTCTGACATCC-3') (SEQ
25 ID NO: 14).

[080] Construction of pHB7 was performed from *P. pastoris* cloning/expression vector pPICZ α B, and the DNA fragment encoding the regions of G3-G5 from pHB6 digestion with *Eco*RI/*Xba*I and agarose purified. The ligation reaction, transformation, restriction analysis and DNA sequencing were all
30 conducted as stated above for pHB6.

[081] Production of pHB8 containing laminin-5- α 3 plasmid G1-G3 domains in pPICZ α B was by removal of the G4 and G5 domain from the plasmid pHB6. This was performed by digestion of pHB6 with *Xba*I enzyme, treated with T4 DNA polymerase to blunt the 3' overhangs, precipitated, followed by a partial digest with *Pvu*II and agarose purification of a 5304 bp fragment. This linear fragment was religated for 24 hours at room temperature and transformed into *E.coli* strain JM109. Construction of plasmid pHB8 was verified by restriction analysis and DNA sequencing as stated for pHB6.

[082] Construction of plasmid pHB9 included the G3 domain of laminin-5- α 3 chain cloned into the expression vector pPICZ α B by the following: digestion of the vector with *Xba*I, treated with T4 DNA polymerase to blunt 3' overhangs, precipitated, digested with *Eco*RI, and agarose purified. The G3 insert was produced from plasmid pHB7 digested with *Eco*RI, precipitated, digested with *Pvu*II and agarose purified. Ligation conditions were 24 hours at room temperature and transformed into *E.coli* strain JM109. Verification of correct construction was as stated for pHB6.

Expression in *P. pastoris*

[083] The *P. pastoris* yeast strain SMD1168 was transformed by the *Pichia* EasyComp™ Kit as described from Invitrogen with 5 μ g of *Sac*I linearized expression plasmids. Multicopy recombinants were selected on YPDS (yeast extract with peptone, dextrose and sorbitol) plates containing 100 μ g/ml Zeocin™. Loss of the AOX1 gene results in a strain that is referred to as Mut^s, which is designated to describe the phenotype of such mutants that lack the ability to metabolize methanol. Cells described as the Mut⁺ phenotype are capable of utilizing methanol as the sole carbon source. These phenotypes are commonly used to evaluate the *P. pastoris* transformants for correct integration into the genome. Transformants were screened for their ability to grow on histidine-deficient minimal dextrose agar plates which confirmed the Mut⁺ phenotype.

Analysis of recombinant laminin

[084] For verification of gene integration into the *P. pastoris* genome, genomic DNA was isolated and analyzed by PCR. Single colonies from the YPDS-Zeocin™ plates were used to inoculate 5 ml overnight cultures in YPD (1% yeast extract, 2% peptone, 2% dextrose) medium. The isolation of genomic DNA was performed by the Rather Rapid Genomic Prep protocol (Hoffman and Winston, Rather Rapid Genomic Prep, *Gene*, Vol. 87:262-272, 1987). Direct PCR screening of *P. pastoris* clones was carried out using the synthetic oligonucleotides encoding the 5' AOX1 (942 bp fragment containing the AOX1 promoter that allows methanol-inducible, high level expression in *P. pastoris*) and 3' AOX1 regions using the same primers described previously in the expression vector construction.

High-cell density expression of recombinant *P. pastoris* strains

[085] Expression of the recombinant laminin protein was carried out in a BioFlo 110 Modular Benchtop Fermentor (New Brunswick Scientific) with a total volume of 2.0 L. The dissolved oxygen was kept at 30% of saturation until feed phase, this was maintained at > 40%. The dissolved oxygen was controlled by the agitation rate. Total inlet gas flow was kept >2 vvm (v=volume of air in ml, v=per unit of medium in L, m=per unit of time in hours). The pH was maintained at 5.0 by the addition of NH₄OH which also served as a minor nitrogen source. The temperature was constant during the fermentation at 30°C.

[086] The inoculum was prepared from 1 ml pre-cultures stored at -80°C in glycerol with an OD₆₀₀ of 35.0. This was resuspended in 5 ml of 1% BMGY medium and incubated at 30°C for 4 hours at 250 rpm. Media containing 1% BMGY includes 1% glycerol, 100 mM potassium phosphate, pH 6.0, 1.34% YNB (yeast nitrogen base) and 4 X 10⁻⁵ % biotin. The culture was used to inoculate 100 ml of overnight medium for an initial OD₆₀₀ of 0.5. Overnight medium was 1% BMGY with the addition of 100 µg/ml of Zeocin™. Incubation continued using the same conditions as before for 11 hour growth. The batch phase was the initial phase in the BioFlo fermentor using 900 ml of batch medium with the addition of the 100 ml inoculum.

Batch medium (1 L) consisted of 12 g glycerol, 100 ml 10X YNB (13.4% yeast nitrogen base with ammonium sulfate without amino acids), 100 ml 1 M potassium phosphate, pH 6.0, 2 ml 500X biotin, 1 ml 1000X trace metals, and 786 ml milliQ water. The batch phase lasted 20 hours. During the glycerol fed-batch phase over the next 18 hours, a continuous feed of 444 g glycerol, 100 ml 10X YNB, 1 ml 1000X trace metals, 2 ml 500X biotin and antifoam were added. The total volume of medium added was 474 ml. The methanol fed-batch phase (induction) continued for 20 hours with a medium volume of 203 ml added. This induction medium was comprised of 200 ml 100% MeOH, 1 ml 1000X trace metals and 2 ml 500X biotin.

Trace metal components for all media above contained the following per liter: 2.0 g CuSO_4 , 0.1 g KI, 3.0 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.2 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.02 g boric acid, 0.5 g CoCl_2 , 7.0 g ZnCl, 10.0 g $\text{FeSO}_4 \cdot \text{H}_2\text{O}$.

Purification of recombinant laminin

[087] The supernatant containing recombinant laminin-5 G domains was collected by centrifugation at 5000 x g for 10 min. Purification chromatography was performed using 10 g of Sephadex™ G-75 superfine (20-50 micron particle diameter) suspended in 500 ml of 0.1 M ammonium acetate pH 6.95. The suspension was allowed to equilibrate overnight. The swelled gel was degassed for 4 hours and packed into a 1 cm X 40 cm Pharmacia column equipped with plastic frit, 10 μ filter disk and flow valve. The gel was allowed to settle at full flow rate under gravity. The column was packed to a height of 28 cm with the G-75. Void volume was determined using 3 ml of 2 mg/ml blue dextran ($\text{MW} > 2 \times 10^6$ Daltons) in PBS containing 1% glycerol. The sample was applied by underlayering and the column was eluted with 0.1 M ammonium acetate until the blue dextran began to emerge (25 ml) and continued until all of the blue dextran had washed from the column (31 ml total). The sample containing 2.5 mg/ml of protein in a 2 ml supernatant plus 1 ml of ammonium acetate was applied to the column as given above. The void volume was obtained as above followed by an additional total column volume of 85 ml that was collected containing the desired recombinant

protein of laminin-5. This sample was freeze dried, resuspended in 1 ml total volume of ddH₂O and used for functional assays.

Example 2

[088] Adhesion characteristics of MDA-MB-435 breast cancer cells to
5 recombinant (*r*) rat laminin-5 α 3 chain G3 domain protein (SEQ ID NO:6) were examined.

[089] Initially, untreated 96-well plates were coated overnight at 4°C with purified recombinant rat laminin-5 α 3 chain G3 domain protein (SEQ ID NO:6). Various concentrations of the G3 domain protein were added to triplicate wells.
10 Specifically, concentrations examined included 0.1, 0.5, 1.0, 2.5, 5.0, 7.5 and 10 μ g/ml diluted in sterile PBS. Following overnight coating, wells were washed twice with PBS and blocked for 1 hour at room temperature with 1% BSA/PSA.

[090] MDA-MB-435 breast cancer cells were collected by brief trypsinization, washed twice with culture medium, and plated in the prepared wells including control
15 wells that had not been coated with the G3 domain protein, at 5×10^5 cells/well. Plates were incubated for 1 hour in a 37°C humidified incubator (5% CO₂). After incubation, wells were washed with medium twice to remove unbound cells, followed by fixing attached cells with 3.7% paraformaldehyde/PBS for 10 minutes, and then staining attached cells with 0.5% crystal violet solution. At the end of 10 minutes
20 staining, wells were washed twice with ddH₂O to remove excess dye followed by addition of 1% SDS to solubilize cells. The amount of crystal violet incorporated into attached cells was determined using a Molecular Devices plate reader set to absorb at 550 nm.

[091] Figure 6 graphically illustrates the results. Mean O.D. readings labeled
25 on the graph with different letters are significantly different at $P \leq 0.0001$.

Example 3

[092] Well plates were coated overnight at 4°C with purified recombinant rat laminin-5 α 3 chain G3 domain protein (SEQ ID NO:6) as described above with all coated wells coated at 5.0 μ g/ml diluted in sterile PBS. Following overnight coating,

wells were washed twice with PBS and blocked for 1 hour at room temperature with 1% BSA/PSA.

[093] MDA-MB-435 breast cancer cells were collected by brief trypsinization and washed twice with culture medium. The MDA-MB-435 cells were split into three portions, one portion was plated as described above in Example 2 at 5×10^5 cells/well in wells previously coated with 5.0 $\mu\text{g/ml}$ G3 domain protein (labeled G3 on Figure 7) and also plated into otherwise untreated well plates (labeled Control on Figure 7). The other two portions were incubated prior to plating with either anti- $\alpha 6$ integrin monoclonal antibody or mouse IgG2a isotope control at a 1:5 dilution for 15 minutes. Following incubation, these portions were also plated in wells previously coated with 5 $\mu\text{g/ml}$ of the G3 protein according to the process described above in Example 2 at 5×10^5 cells/well (labeled G3+Anti- $\alpha 6$ and G3+IgG2a, respectively, on Figure 7) and also were plated in untreated well plates, i.e., wells not previously coated with the G3 protein (labeled Anti- $\alpha 6$ and IgG2a on Figure 7, respectively).

[094] All plates were incubated for 1 hour in a 37°C humidified incubator (5% CO_2). After incubation, wells were washed with medium twice to remove unbound cells, followed by fixing attached cells with 3.7% paraformaldehyde/PBS for 10 minutes, and then staining attached cells with 0.5% crystal violet solution. At the end of 10 minutes staining, wells were washed twice with ddH₂O to remove excess dye followed by addition of 1% SDS to solubilize cells. The amount of crystal violet incorporated into attached cells was determined using a Molecular Devices plate reader set to absorb at 550 nm.

[095] Figure 7 graphically illustrates the results. Mean O.D. readings labeled on the graph with different letters are significantly different at $P \leq 0.0001$.

Example 4

[096] Purified recombinant rat laminin-5 $\alpha 3$ chain G3 domain protein (SEQ ID NO:6) was added in increasing concentrations to wells of 96-well plates as described above. Specifically, the concentrations of G3 domain protein added to wells were 1.0, 2.5, 5.0, 7.5, 10 and 15 $\mu\text{g/ml}$ diluted in culture medium.

[097] MDA-MB-435 cells were collected by brief trypsinization, washed twice with culture medium, and plated at 5×10^5 cells/well in the prepared wells as well as in control wells not coated with the G3 domain protein. Plates were incubated for 24 hours at 37°C in a humidified incubator (5% CO₂). Twenty µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent (5 mg/ml) was added to all wells during the final 4 hours of incubation. One hundred µl of culture supernatant was removed from each well followed by the addition of 100 µl dimethylsulfoxide (DMSO) to solubilize the cells. Plates were shaken for 15 minutes and absorbance was recorded at dual wavelengths of 570/650 nm using a Molecular Devices plate reader to examine the proliferation of the cancer cells in the wells.

[098] Figure 8 illustrates the results. Mean O.D. readings labeled on the graph with different letters are significantly different at $P \leq 0.0001$. As can be seen, cancer cell proliferation declined with increasing dosage level of the G3 domain protein composition.

Example 5

[099] Well plates were coated overnight at 4°C with purified recombinant rat laminin-5 α3 chain G3 domain protein (SEQ ID NO:6) as described above with all coated wells coated at 10.0 µg/ml diluted in sterile PBS. Following overnight coating, wells were washed twice with PBS and blocked for 1 hour at room temperature with 1% BSA/PSA.

[0100] MDA-MB-435 breast cancer cells were collected by brief trypsinization and washed twice with culture medium. The MDA-MB-435 cells were split into three portions, one portion was plated as described above in Example 4 at 5×10^5 cells/well in wells previously coated with 10.0 µg/ml G3 domain protein (labeled G3 on Figure 9) and also plated into otherwise untreated well plates (labeled Control on Figure 9). The other two portions were incubated prior to plating with either anti-α6 integrin monoclonal antibody or mouse IgG2a isotope control at a 1:10 dilution for 15 minutes. Following incubation, these portions were plated in wells previously coated with 10 µg/ml of the G3 protein according to the process described above in Example 2 at 5×10^5 cells/well (labeled G3+Anti-α6 and G3+IgG2a, respectively,

on Figure 9) and also were plated in untreated well plates, i.e., wells not previously coated with the G3 protein (labeled Anti-alpha6 and IgG2a on Figure 9, respectively).

5 [0101] Plates were incubated for 24 hours at 37°C in a humidified incubator (5% CO₂). Twenty µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent (5 mg/ml) was added to all wells during the final 4 hours of incubation. One hundred µl of culture supernatant was removed from each well followed by the addition of 100 µl dimethylsulfoxide (DMSO) to solubilize the cells. Plates were shaken for 15 minutes and absorbance was recorded at dual
10 wavelengths of 570/650 nm using a Molecular Devices plate reader to examine the proliferation of the cancer cells in the wells.

[0102] Figure 9 illustrates proliferation results. Mean O.D. readings labeled on the graph with different letters are significantly different at $P \leq 0.0001$. As can be seen, proliferation declined with the G3 domain protein and with the Anti-x 6
15 antibody, with the combination of the two exhibiting the best results.

[0103] It will be appreciated that the foregoing examples, given for purposes of illustration, are not to be construed as limiting the scope of this invention. Although only a few exemplary embodiments of this invention have been described in detail above, those skilled in the art will readily appreciate that many modifications
20 are possible in the exemplary embodiments without materially departing from the novel teachings and advantages of this invention. Accordingly, all such modifications are intended to be included within the scope of this invention which is defined in the following claims and all equivalents thereto. Further, it is identified that many embodiments may be conceived that do not achieve all of the advantages
25 of some embodiments, yet the absence of a particular advantage shall not be construed to necessarily mean that such an embodiment is outside the scope of the present invention.